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Characterization of two chitin synthase genes of the red flour beetle, *Tribolium castaneum*, and alternate exon usage in one of the genes during development☆

Yasuyuki Arakane [a], David G. Hogenkamp [b], Yu Cheng Zhu [c], Karl J. Kramer [a,b], Charles A. Specht [d], Richard W. Beeman [a], Michael R. Kanost [b], Subbaratnam Muthukrishnan [b,*]

[a] *Grain Marketing and Production Research Center, ARS-USDA, 1515 College Avenue, Manhattan, KS 66502, USA*
[b] *Department of Biochemistry, 104 Willard Hall, Kansas State University, Manhattan, KS 66506-3702, USA*
[c] *Jamie Whitten Delta States Research Center, ARS-USDA, Stoneville, MS 38776, USA*
[d] *Department of Medicine, Section of Infectious Diseases, Boston University, Boston, MA 02118, USA*

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Abstract

Two chitin synthase (CHS) genes of the red flour beetle, *Tribolium castaneum*, were sequenced and their transcription patterns during development examined. By screening a BAC library of genomic DNA from *T. castaneum* (Tc) with a DNA probe encoding the catalytic domain of a putative *Tribolium* CHS, several clones that contained *CHS* genes were identified. Two distinct PCR products were amplified from these BAC clones and confirmed to be highly similar to *CHS* genes from other insects, nematodes and fungi. The DNA sequences of these genes, *TcCHS1* and *TcCHS2*, were determined by amplification of overlapping PCR fragments from two of the BAC DNAs and mapped to different linkage groups. Each ORF was identified and full-length cDNAs were also amplified, cloned and sequenced. *TcCHS1* and *TcCHS2* encode transmembrane proteins of 1558 and 1464 amino acids, respectively. The *TcCHS1* gene was found to use alternate exons, each encoding 59 amino acids, a feature not found in the *TcCHS2* gene. During development, *Tribolium* expressed *TcCHS1* predominantly in the embryonic and pupal stages, whereas *TcCHS2* was prevalent in the late larval and adult stages. The alternate exon 8a of *TcCHS1* was utilized over a much broader range of development than exon 8b. We propose that the two isoforms of the TcCHS1 enzyme are used predominantly for the formation of chitin in embryonic and pupal cuticles, whereas TcCHS2 is utilized primarily for the synthesis of peritrophic membrane-associated chitin in the midgut.
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1. Introduction

Many tons of chitin, a homopolymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc), are recycled annually in the biosphere with fungi and arthropods being the principal producers (Muzzarelli, 1999). Chitin is synthesized from cytoplasmic pools of UDP–*N*-acetylglucosamine (UDP–GlcNAc) by chitin synthases (CHSs) (EC 2.4.1.16) located in the plasma membrane, which also ensure that the polymer is extruded outside of the cell into an extracellular matrix. In many species

Abbreviations: Tc, *Tribolium castaneum*; CHS, chitin synthase; UDP, uridine-5′-diphosphate; GlcNAc, *N*-acetylglucosamine; RT, reverse transcriptase; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl and 0.015 M sodium citrate pH 8.0.

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* Corresponding author. Tel.: +1-785-532-6939; fax: +1-785-532-7278.

E-mail address: smk@ksu.edu (S. Muthukrishnan).

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of fungi, chitin is an essential component of the cell wall and septum, whereas for insects, it is an essential component of the exoskeleton and the peritrophic membrane (PM) that lines the midgut. CHSs have been identified in a variety of organisms including nematodes, fungi and insects. Amino acid sequence similarities have been the principal tools used for identifying CHSs, which form a subfamily within a larger group (family 2) of the glycosyltransferases (Coutinho and Henrissat, 1999).

Fungal CHSs have been studied extensively and various ones were found to function at different developmental stages such as cell division, sporulation and hyphal branching (Merz et al., 1999; Henar Valdivieso et al., 1999; Munro and Gow, 2001; Roncero, 2002). Consistent with their multifarious roles, fungal CHSs are encoded by a large family of genes and as many as eight different enzymes have been identified in a single species.

In contrast to fungi, insects and nematodes have fewer genes encoding CHSs (Zhu et al., 2002). The number of *CHS* genes in most insect species is likely to be two, based upon sequencing of the *Drosophila* and *Anopheles* genomes (*Drosophila* and *Anopheles* Genome Project databases at the National Center for Biotechnology Information). Genome sequencing of the nematode, *Caenorhabditis elegans*, also revealed two *CHS* genes (Veronico et al., 2001). Individual *CHS* genes from the blow fly (*Lucilia cuprina*), mosquito (*Aedes aegypti*), and tobacco hornworm (*Manduca sexta*) have been described recently based on cDNA sequencing, and preliminary evidence suggests the existence of a second gene in each of these species (Tellam et al., 2000; Zhu et al., 2002; our unpublished data). *CHS* genes have been reported for three other species of nematodes, *Brugia malayi*, *Meloidogyne artiellia*, and *Dirofilaria immitis* (Harris et al., 2000; Veronico et al., 2001; Harris and Fuhrman, 2002), although it remains unclear whether any of these organisms have more than one *CHS* gene.

It has been suggested that insects have two CHSs, one responsible for synthesis of cuticular chitin and the other dedicated to the synthesis of chitin associated with the peritrophic membrane (Tellam et al., 2000; Zhu et al., 2002). However, to verify this suggestion, an extensive characterization of all *CHS* genes and their expression in different tissues and developmental stages in a single insect species must be completed. In this paper, we report the isolation and characterization of two distinct genes for CHS from the red flour beetle, *Tribolium castaneum*. We also demonstrate that even though there are only two *CHS* genes, *Tribolium* can generate different enzymes or isoforms as a result of alternate exon usage. This process appears to be developmentally regulated.

2. Materials and methods

2.1. Insect growth, developmental stages and BAC libraries

Beetles were reared at 30 °C in whole wheat flour fortified with 5% (v/v) Brewer's yeast under standard conditions (Beeman and Stuart, 1990). The following life stages were used for analysis of *CHS* gene expression during development: embryos (0–3 days post-oviposition), early larvae (actively feeding, 6th–7th instar), last instar larvae (actively feeding, 7th–8th instar), prepupae (quiescent, post-feeding 7th–8th instar), pupae and adults. Instar numbering in *Tribolium* is indeterminant and cannot be known with certainty when mass culturing. The BAC library (a gift from Exelixis Pharmaceutical Co., South San Francisco, CA) was prepared in the plasmid vector pBACe3.6 using a partial *Eco*RI digest of genomic DNA from the highly inbred GA-2 strain. At the time of screening, the library consisted of approximately 8400 clones with an average insert size of about 129 kb and a genome coverage of ca. 5.4×.

2.2. Preparation of a DNA probe for TcCHS catalytic domain

To obtain a DNA probe for the identification of BAC clones containing *TcCHS* genes, we designed a pair of degenerate primers (forward: 5′-TTYGARTAYGCNATHGGNCAYTGG-3′ and reverse: 5′-CCANCKRTCYTCNCCYTGRTCRTAYTG-3′) corresponding to two highly conserved amino acid sequences in the catalytic domain of CHSs, FEYAIGHW and QYDQGEDRW, respectively. These primers were used to amplify a 242 bp PCR product using *Tribolium* genomic DNA as the template. The PCR product was cloned into the pGEM-T vector (Promega). The cloned DNA was sequenced and shown to represent a *CHS* gene fragment by using the BLASTX search program of the GenBank protein database. The insert DNA from this clone was used subsequently for screening the *Tc* BAC library.

2.3. Screening of Tc BAC library with CHS DNA probe

The PCR fragment with sequence similarity to *CHS* genes obtained as described in Section 2.2 was labeled with ^{32}P-dATP using the Promega primer-labeling kit and used as a probe for screening the *Tribolium* BAC library. A nylon membrane was double-loaded (diagonally or side by side) with a total of approximately 8400 colonies representing the *Tc* BAC library. The membrane was hybridized with the ^{32}P-labeled probe in PerfectHyb Plus buffer (Sigma) at 62 °C for 18 h. The

membrane was washed at 55 °C for 1 h with three changes of 0.2× SSC/0.1% SDS and exposed to X-ray film to identify colonies that hybridized either strongly or weakly with the probe.

2.4. Determination of the DNA sequences of TcCHS genes

BAC plasmids containing the *TcCHS* genes were prepared by the alkaline-SDS method and used as templates for PCR amplification of different regions of the *TcCHS* genes. The sequences of degenerate primers and their locations in the *TcCHS* genes are shown in Table 1A and Fig. 1, respectively. For amplification of 5′- and 3′-end sequences, single primers, SF3, SR3, and WF3 (see Table 1B for sequences) were used. To obtain the 5′-end sequence of *TcCHS2*, inverse PCR was conducted using gene-specific primers, WF4 and WR4 (Table 1B). Approximately 2 μg of BAC DNA containing the *TcCHS2* gene were digested with restriction enzyme *Pst*I and the fragments were purified by phenol–chloroform extraction followed by ethanol precipitation. T4 DNA ligase was added to the fragments to allow the formation of circular DNA as a result of self-ligation. PCR reactions were conducted in a final volume of 50 μl containing 20 mM Tris–HCl, pH 8, 50 mM KCl, 2 mM $MgCl_2$, 0.4 μM of the primers, 0.2 mM dNTPs, 2.5 units of Ex Taq™ polymerase and approximately 10 ng of the plasmid template. PCR reactions were conducted for 25 cycles with 1 min of denaturation at 94 °C, 1 min of annealing at 42–60 °C, and 3 min of extension at 72 °C. The amplified DNA fragments were subcloned into the pGEM-T vector (Promega) and sequenced using flanking vector primers T7 and SP6 and/or gene specific primers (Sequencing and Genotyping Facility, Kansas State University).

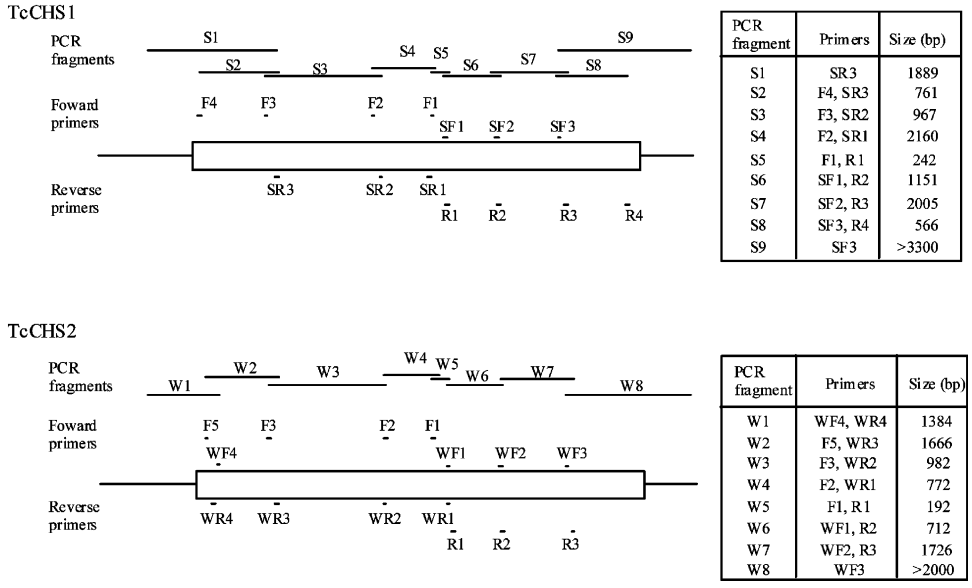
2.5. Cloning of cDNAs for CHS genes

To obtain the cDNAs corresponding to the entire protein coding regions of *TcCHS1A*, *TcCHS1B* and *TcCHS2*, mRNAs were purified from prepupae using the Oligotex Direct mRNA Kit (Qiagen) according to the manufacturer's instructions. Reverse transcriptions were performed with SUPERSCRIPT II RNase H-Reverse Transcriptase (Invitrogen) using an oligo-(dT) primer. The primers used were 5′-ATGACATCCGGG-GGGCTG-3′ and 5′-TCACATCCTGCTATTGCTGC-3′ for *TcCHS1*, and 5′-ATGGCGGCGCGTCATCG-3′ and 5′-TTATGCCTCCACGTCTGACC-3′ for *TcCHS2*. PCR reactions were conducted as follows: denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and polymerization at 72 °C for 4.5 min using Takara LA *Taq* polymerase for 30 cycles. The products of each reaction were subjected to electrophoresis on 0.8% agarose gel containing 1 μg/ml crystal violet (Invitrogen), excised and purified using the Freeze 'N Squeeze DNA Gel Extraction Spin Column (Bio-Rad).

Table 1
Primers for the amplification of genomic DNA containing *TcCHS1* and *TcCHS2*

Name	Primer sequence (5′-3′)	Conserved AA sequence
(A) Degenerate primers		
Forward primer		
F1	TTY GAR TAY GCN ATH GGN CAY TGG	FEYAIGHW
F2	TGY GCN ACN ATG TGG CAY G	CATMWHE
F3	GGN TGG TGG GAR AA	GW WEN
F4	CAR GAR ACN AAR GGN TGG GA	QETKGWE
F5	TGG GAY GTN TTY MGN GAY CCN CC	WD VFDDPP
Reverse primer		
R1	CCA NCK RTC YTC NCC YTG RTC RTA YTG	QYDQGEDRW
R2	ACY TCN CKN GTN CCC CA	WGTRE
R3	AAN CKR TGR AAN ARC ATN GC	AMLFHRF
R4	TTN GCN CCN TRN GTY TGC AT	MQTQ/YGAR

Name	Primer sequence (5′-3′)	Name	Primer sequence (5′-3′)
(B) Gene-specific primers			
Forward primer			
SF1	CGG AAG CCA GGC ATT ACG TG	WF1	CCC AAG CCA AGC ACT ACG TG
SF2	TGC TAC TGA TTT TGT ACT CC	WF2	TCC TCC TAG TCA TCT ACT CG
SF3	TGG AAC CGA TCG GTC TAG TC	WF3	CCA TCG GTT TCG TGT TCC TC
		WF4	TTT GTG GTC CTT GGT GC
Reverse primer			
SR1	CAT GCT CGG TGG CCT TTT GC	WR1	CGT GCT CGG TGG CTT TTT GC
SR2	CCA GTC GCA AAA TCG ACT TC	WR2	CCA AGC GGA ACA CCG ATT TC
SR3	TTC TCC TTG ATT TTG CCC AG	WR3	AAC TCC TTT TTG CTC TTG CC
		WR4	CAC AGT TAT GAT TGT CAC AAC



PCR fragment	Primers	Size (bp)
S1	SR3	1889
S2	F4, SR3	761
S3	F3, SR2	967
S4	F2, SR1	2160
S5	F1, R1	242
S6	SF1, R2	1151
S7	SF2, R3	2005
S8	SF3, R4	566
S9	SF3	>3300

PCR fragment	Primers	Size (bp)
W1	WF4, WR4	1384
W2	F5, WR3	1666
W3	F3, WR2	982
W4	F2, WR1	772
W5	F1, R1	192
W6	WF1, R2	712
W7	WF2, R3	1726
W8	WF3	>2000

Fig. 1. (A) Strategies for cloning and sequencing genomic DNA fragments encoding *Tribolium castaneum* chitin synthases. The fragments containing *TcCHS1* and *TcCHS2* sequences were amplified by PCR using degenerate/gene-specific primer combinations except for fragments S1, S9, W1 and W8. The fragments S1, S9 and W8 were amplified using a single gene specific primer. The fragment W1 was obtained by inverse PCR. The positions of the PCR fragments, primer combinations used to generate them, and the sizes of the PCR products are indicated on the right.

The purified fragment was cloned into a pCR-XL-TOPO vector (Invitrogen). Furthermore, to identify the cDNAs of *TcCHS1A* and *TcCHS1B*, colony PCR was done using the common forward primer designed based on exon 6, 5′-TGCTACTGATTTTGTACTCC-3′, and either of the following exon 8a- or exon 8b-specific reverse primers: 5′-TTGGGTGCTCTCGTCATAG-3′ for exon 8a and 5′-CGGACGTTTCCTCAATATAC-3′ for exon 8b. Three cloned cDNA fragments corresponding to *TcCHS1A*, *TcCHS1B*, and *TcCHS2* were fully sequenced using appropriate primers. Furthermore, to obtain the 5′ end of cDNAs of *TcCHS1* and *TcCHS2*, 5′-RACE was performed using the 5′-RACE system version 2.0 (Invitrogen) according to the manufacturer's instructions. The following antisense gene-specific primers were used for the synthesis of the first strand cDNAs from total RNA of pupae and larvae: 5′-TTCTCCTTGATTTTGCCCAG-3′ (spanning positions 850 and 869) for *TcCHS1* and 5′-AACTCCTTTTTGCTCTTGCC-3′ (spanning positions 823 and 842) for *TcCHS2*. Following the addition of a homopolymeric C-tail at the 3′-end of the cDNAs, PCR was carried out using an adapter primer and the gene-specific primers: 5′-TTGACGGTGATTTC-GAGGC-3′ (spanning positions 185 and 203) for *TcCHS1* and 5′-CACAGTTATGATTGTCACAAC-3′ (spanning positions 184 and 204) for *TcCHS2*. To obtain the 3′-ends of mRNAs, PCR was done using an oligo-(dT) adapter primer and the gene-specific forward primers, 5′-TGACCATGAGGAGAGAG-3′ (spanning positions 4382 and 4398) for *TcCHS1* and 5′-TAGACTTCGATCTGTGCAGC-3′ (spanning positions 3956 and 3975) for *TcCHS2* and first strand cDNAs as the template.

2.6. Analysis of expression of TcCHS1 and TcCHS2 by RT-PCR during development

Total RNA was isolated from whole insects of different developmental stages and from male and female pupae and adults (see Section 2.1) using the RNeasy Mini Kit (Qiagen). Two micrograms of total RNA were used as templates for first strand cDNA synthesis using an oligo-(dT) primer. This cDNA was used then as the template for amplification and detection of specific *TcCHS* sequences. The primers used were 5′-TGCTACTGATTTTGTACTCC-3′ (spanning positions 3131 and 3150 in exon 6) and 5′-TCA-CATCCTGCTATTGCTGC-3′ (spanning positions 4661 and 4677 in exon 10) for *TcCHS1*, and 5′-TCCTCCTAGTCATCTACTCG-3′ (spanning positions 3050 and 3069 in exon 6) and 5′-TTATGCCTC-CACGTCTGACC-3′ (spanning positions 4376 and 4395 in exon 8) for *TcCHS2*. These primers were chosen to achieve discrimination between *CHS1* and *CHS2* transcripts and to generate products of different sizes using a single PCR reaction containing a mixture of all four primers and cDNA as the template.

2.7. Analysis of TcCHS1 transcripts for alternate exon usage

The presence of *TcCHS1* transcripts containing exon 8a or 8b in total RNA was analyzed using a PCR assay with primer combinations designed to discriminate between the two exons. cDNAs prepared from total RNA isolated from different stages of development were used as the template. A 20-nucleotide-long primer, 5′-TGCTACTGATTTTGTACTCC-3′ (1F) in exon 5 of *TcCHS1*, was the forward primer. The reverse primer (3R) for exon 8a was 5′-TTGGGTGCTCTCGTCATAG-3′ and the primer for exon 8b (4R) was 5′-CGGACGTTTCCTCAATATAC-3′. PCR reactions were conducted using the following conditions: denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and polymerization at 72 °C for 1 min for 23 cycles. The products of each reaction were fractionated by electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide.

2.8. DNA and protein sequence analyses

The *CHS* genes of *Drosophila* and *Anopheles* were analyzed to determine probable exons and introns of each gene. Initially, a series of "TBLASTN" searches of the *Drosophila* and *Anopheles* Genome Project databases at NCBI were made by using as queries the protein sequences of *M. sexta* CHS1 (AAL38051), *L. cuprina* CHS1 (AAG09712), *A. aegypti* CHS1 (AAF34699) and the protein sequences for TcCHS1 and TcCHS2 as determined in this study. Exons were chosen that translated to protein sequences most similar to the queries and introns that had conventional 5′ and 3′ splice sites. Exons and introns were subsequently confirmed by recently submitted full-length cDNA sequences of *DmCHS1* (NM_169052 and NM_079509), *DmCHS2* (NM_079485) and *AgCHS2* (XM_321336 and XM_321337); and a partial cDNA sequence of *AgCHS1* (XM_321951). The presence of alternate exons corresponding to exon 8 of *TcCHS1* in *M. sexta CHS1* was confirmed by sequencing of a PCR-amplified genomic fragment (Hogenkamp et al., unpublished data).

Alignment of nucleotide sequences and deduced amino acids from cDNA clones was made using ClustalW software (PAM250). Protein sequences were analyzed for transmembrane helices using the TMHMM v.2.0 software available at http://www.cbs.dtu.dk/services/TMHMM-2.0/. Coiled-coil domains were identified using the Paircoil Program which can be accessed at http://theory.lcs.mit.edu/~bab/paircoil/paircoil.html. Multiple sequence alignments to yield phylogenetic trees were generated with Megalign Software (DNAStar, Madison, WI) using ClustalW (PAM250).

2.9. Mapping of TcCHS genes to specific Tribolium linkage groups

CHS-positive BACs were genetically mapped by single-strand conformation polymorphism (SSCP) analysis. We identified SSCP dimorphisms between two highly inbred *T. castaneum* strains, GA-2 and ab2, using primer pairs specific for end-sequences from each BAC. The BACs were mapped onto a whole-genome recombination map at an average resolution of ca. 1.5 cM using a backcross family that consisted of 179 siblings and using a marker set totaling more than 400 unique DNA sequences derived from BACs, cDNAs and other sources. Details of this mapping procedure will be published elsewhere (Beeman et al., unpublished data).

2.10. Southern blot analysis of Tribolium genomic DNA for CHS sequences

Tribolium genomic DNA (10 μg per sample) was digested with five different restriction enzymes, *Ase*I, *Bam*HI, *Cla*I, *Hin*cII, *Hin*dIII and *Xba*I and separated on 0.9% agarose gel. Nucleic acids were transferred onto Hybond N^+ Nylon membrane (Amersham) under alkaline condition and hybridized with random primed ^{32}P-labeled probes (Ready-To-Go DNA Labeling Beads, Amersham). The probe was the 506 bp PCR fragment spanning nucleotide positions 2233 through 2739 of *TcCHS1* cDNA. The membrane was hybridized to the probe at 50 °C and washed at 37 °C with 1× SSC/0.1% SDS and exposed to X-ray film for 3 d.

3. Results

3.1. Screening of Tc BAC library with the CHS probe

A 242 bp PCR fragment amplified from *T. castaneum* genomic DNA as outlined in Section 2.2 using two degenerate primers derived from two highly conserved regions in other insect chitin synthase genes was labeled with ^{32}P-dATP and used to screen an ordered BAC library of *Tc* genomic DNA. Sixteen colonies hybridized to the probe, 12 strongly and four weakly. Plasmid DNA preparations were made from 12 of these BAC colonies. PCR amplification using these BAC DNAs as templates and one degenerate forward primer corresponding to FEYAIGHW and another reverse primer corresponding to another conserved block of residues, QYDQGEDRW, found in the catalytic domains of other insect CHSs (Tellam et al., 2000; Ibrahim et al., 2000, Zhu et al., 2002; Zimoch and Merzendorfer, 2002; Gagou et al., 2002; Ostrowski et al., 2002), yielded amplification products with sizes of either 242 or 192 bp. The DNA from all eight colonies

with strong hybridization to the probe yielded the 242 bp product, whereas the four colonies with the weaker hybridization yielded the 192 bp PCR product. Sequencing of these fragments from four strongly hybridizing and four weakly hybridizing BAC clones indicated that the sequences of all of the members within the same size group were identical except in the degenerate primer regions. The DNA sequence of the larger PCR product was similar to the smaller PCR product (58.1% identity) with the additional 50 bp subsequently confirmed to be an intron. The encoded peptide sequences of both PCR fragments (see underlined sequences in Fig. 3) were highly similar to a conserved region found in insect and nematode CHSs. At this point, the gene that generated the 242 bp fragment was named *TcCHS1* and that which amplified the 192 bp fragment was named *TcCHS2*.

3.2. Sequence and organization of TcCHS1 and TcCHS2 genes

One BAC clone containing the *TcCHS1* gene and a second clone containing the *TcCHS2* gene were chosen for the determination of the complete sequences of these genes. These two DNAs were used as templates in separate PCR reactions with different combinations of degenerate primers corresponding to conserved regions of insect/nematode CHSs. The designations of the primers, the sizes of the PCR products, and their relationship to the corresponding genes are indicated in Fig. 1. The sequences of the degenerate and gene-specific primers as well as the corresponding conserved amino acid sequences are shown in Table 1. In some cases, a single gene-specific CHS primer amplified the 5′- or 3′-end fragment presumably via a type of universal PCR strategy, one end of each such amplified fragment having been misprimed. In one case, an inverse PCR reaction was used to obtain the 5′-end sequence extending upstream of the protein-coding region (Fragment W-1). These strategies resulted in a series of overlapping PCR fragments and it was then possible to assemble the DNA sequences of the entire protein coding regions as well as both the 5′- and 3′-flanking sequences.

About 15.6 and 8.1 kb of genomic DNA sequence were determined for *TcCHS1* and *TcCHS*, respectively. By using BLASTX to align these sequences with protein sequences of insect CHSs in the NCBI database, it was possible to predict the ORFs and the exon–intron organization of the *TcCHS1* and *TcCHS2* genes (Fig. 2). For confirmation, we amplified the entire protein coding regions by RT-PCR using prepupal poly(A)-RNA as template, and *TcCHS1*- and *TcCHS2*-specific forward and reverse primers. cDNAs of 4.7 kb and 4.4 kb were amplified corresponding to the ORFs of *TcCHS1* and *TcCHS2*, respectively (data not shown). The *TcCHS1* PCR product was actually a mixture of two sequences as a result of alternate exon splicing (see below). The DNA sequences of these PCR products and the 5′- and 3′-RACE PCR products confirmed the predicted exon–intron boundaries, as well as independently checking the accuracy of comparable sequences generated from the BAC clones. These three cDNA sequences have been deposited in the GenBank database with accession numbers AY291475, AY291476 and AY291477. The *TcCHS1* and *TcCHS2* genomic DNA sequences have been assigned GenBank accession numbers of AY295880 and AY295879, respectively.

The *TcCHS1* gene has 10 exons and nine introns, whereas the *TcCHS2* gene has eight exons and seven introns (Fig. 2). Approximately 1 kb of the promoter region of *TcCHS1* and 1.1 kb of the promoter region of *TcCHS2* have been sequenced. We identified putative TATA boxes upstream of exon 1 and polyadenylation sequences in the 3′-untranslated regions in both genes. The start of translation of *TcCHS1* is in exon 2 (which is preceded by a long intron), whereas it is in exon 1 of *TcCHS2*. It is clear that the organizations of the two genes in *Tribolium* are different. Two introns are in identical positions in the two genes (introns 3 and 8 of *TcCHS1* and introns 4 and 6 of *TcCHS2*, respectively), while others are at variable positions (Figs. 2 and 3). The introns range in size from 46 to 5623 bp. The most interesting difference between the two genes is the presence in tandem of two non-identical copies of exon 8 (denoted as 8a and 8b) in *TcCHS1*, whereas *TcCHS2* has only one copy of the corresponding region as a part of exon 6 (Fig. 2).

Also shown in Figs. 2 and 3 is a comparison of the exon–intron organization of *CHS* genes from *T. castaneum*, together with those of *D. melanogaster* and *A. gambiae*. Both species have one gene related to *TcCHS1* and one related to *TcCHS2*. Presented is a compilation from analysis of genomic sequences from their respective genome projects, using cDNAs available as separate sequence files submitted to GenBank and various BLAST queries to identify exons and introns. It is clear that the organization of insect chitin synthase genes has diverged among the three species compared, which included two dipterans and one coleopteran. Only one intron has remained in the same position in all six *CHS* genes (see Fig. 3). Like *TcCHS1*, both *DmCHS1* and *AgCHS2* have the start of translation in exon 2 and have alternate exons equivalent to exons 8a and 8b of *TcCHS1*. In all three of these genes, the positions of introns that flank the alternate exons are conserved and the exons are identical in length, encoding 59 amino acids. Tellam et al. (2000) previously reported the identification of the alternate exons (7a and 7b) in *D. melanogaster CHS1*. The *Anopheles CHS2* gene, which has high sequence

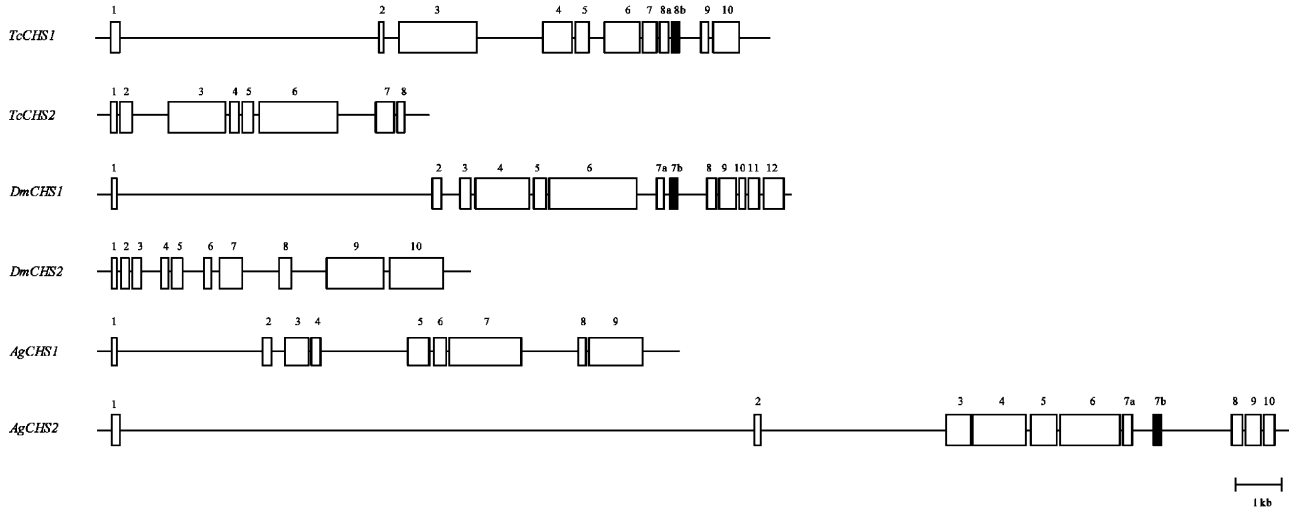


Fig. 2. Schematic diagram of the organization of the *TcCHS1*, *TcCHS2*, *DmCHS1*, *DmCHS2*, *AgCHS1* and *AgCHS2* genes. Boxes indicate exons. Lines indicate introns. The second of the two alternative exons (8b) of *TcCHS1*, *DmCHS1*(7b), and *AgCHS2*(7b) are indicated as closed boxes. About 15.6 kb of the *TcCHS1* and 8.1 kb of *TcCHS2* gDNA sequences were compared to their respective cDNA sequences to define the exons and introns. The exon–intron organization of the other four *CHS* genes was deduced partially from comparisons of available cDNA and genomic sequences but also from the sequence analysis described in Section 2.

similarity with *DmCHS1* and *TcCHS1*, has the equivalent alternate exons 7a and 7b.

The longest ORFs found in the cDNAs were 4674 bp in *TcCHS1* (with either exon 8a or 8b present, see below) and 4392 bp in *TcCHS2*, which are capable of coding for proteins with 1558 and 1464 amino acids, respectively. The amino acid sequence identity between the two proteins, TcCHS1B and TcCHS2, is 47.5% and similarity is 71.0%. The identity and similarity between TcCHS1A and TcCHS2 are 47.8% and 71.1%, respectively. The sequences of these two TcCHS proteins are compared to CHSs from *Drosophila* and *Anopheles* in Fig. 3, which are the only insect species for which both CHS protein sequences are currently available. As with other insect CHSs including those of the sheep blow fly (Tellam et al., 2000), the TcCHSs have an N-terminal domain with several membrane-spanning regions, a central domain with high sequence identity to putative catalytic domains of CHSs of fungi, nematodes and other insects, and a C-terminal domain with multiple membrane spanning regions. TcCHS1 is predicted to have nine transmembrane segments in the N-terminal domain, whereas the TcCHS2 protein is expected to have only five such segments. Using the Paircoil Program (Lupas et al., 1991), a coiled-coil domain is predicted with high probability to occur in TcCHS1 (see Table 2) but not in the TcCHS2 protein.

3.3. Expression of *TcCHS1* and *TcCHS2* during development of *Tribolium*

To determine whether the two *TcCHS* genes are differentially expressed at various stages of development of *Tribolium*, we analyzed cDNA made from RNA isolated at different developmental stages including embryos, early larvae (penultimate larval instar), late larvae (last larval instar), prepupae, pupae and adults by PCR using primers specific for *TcCHS1* and *TcCHS2*. PCR reactions were carried out with each cDNA preparation as the template and the two pairs of PCR primers for *TcCHS1* and *TcCHS2* in the same tube. Control experiments with a mixture of equal amounts of *TcCHS1* and *TcCHS2* cDNAs as templates indicated that the PCR amplification efficiencies were similar with the two sets of primers whose T_ms, lengths and concentrations were nearly the same (bottom panel of Fig. 4B). As shown in Fig. 4A, the expected PCR product (1546 bp) corresponding to fully spliced *TcCHS1* transcripts was detected in embryos, late larvae, prepupae and pupae but not in early instar larvae. Trace amounts of *TcCHS1* transcripts were observed in early larvae and young adults but not in mature adults (Fig. 4C). No PCR products with the size expected for fully spliced transcripts of *TcCHS2* (1345 bp) were seen at the embryonic stage, but they were detected in both early- and late larvae (Fig. 4A, lanes 2 and 3). *TcCHS2* transcripts were not detected at the prepupal and pupal stages. However, in mature adults, only *TcCHS2* transcripts were detected. Although the PCR data are only qualitative, they do suggest differences in the developmental pattern of expression of *TcCHS1* and *TcCHS2* genes. *TcCHS1* is expressed at the pupal stage, whereas *TcCHS2* is expressed at the late larval stage and in mature adults. Similar analyses with RNA preparations from male and female pupae, and

Fig. 3. Alignment of deduced amino acid sequences of TcCHS1, TcCHS2, DmCHS1, DmCHS2, AgCHS1 and AgCHS2 using ClustalW software. Transmembrane regions predicted using TMHMM software (v. 2.0) are shaded. The positions in the protein sequences of TcCHS1 and TcCHS2 where coding regions are interrupted by introns are indicated by shaded arrow heads. Intron 1 of *TcCHS1* lies in the 5′-UTR region two nucleotides 5′ of the translation start and is not indicated in this figure. The putative catalytic domains are boxed. Symbols below the aligned amino acid sequences indicate identical (∗), highly conserved (:), and conserved residues (.). The regions in TcCHS1 and TcCHS2 corresponding to the PCR probe made from two degenerate primers representing two highly conserved sequences in CHSs are underlined.

Table 2
Correlation between the presence of the alternate exon and the coiled-coil domain

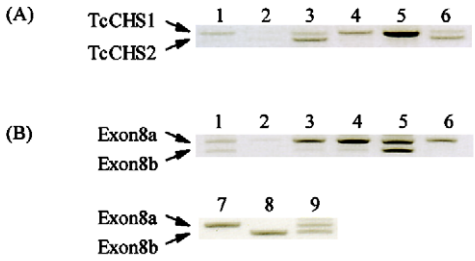
Name	Presence of alternate exon	Coiled-coil domain	
		Probability	Position[a]
Insect			
AaCHS1	No[b]	None	–
AgCHS1	No	None	–
AgCHS2	Yes	High	I
DmCHS1	Yes	High	I
DmCHS2	No	None	–
TcCHS1	Yes	High	I
TcCHS2	No	Low	II
MsCHS1	Yes	High	I
LcCHS1	Yes[b]	High	I

[a] Position I is immediately after the 5-TMS region. Position II is 40–50 amino acid downstream from 5-TMS region.
[b] Prediction based on comparison with other insect CHSs.

young adults and mature adults (Fig. 4C, top panel) indicated that there were no significant differences in expression of the two *CHS* genes between males and females.

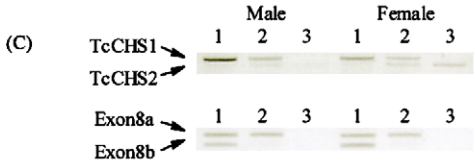
3.4. Alternate exon usage in generation of *TcCHS1* transcripts

The presence of two alternate forms of exon 8 in the *TcCHS1* gene suggests the possibility of alternate exon usage in generating two different transcripts encoding two different TcCHS1 proteins. To investigate whether alternate exon usage occurs, we analyzed cDNA prepared from RNA isolated from *Tribolium* at different stages of development for the presence of exon 8a or 8b sequences of *TcCHS1* using a forward primer in exon 5 and reverse primers specific for either exon 8a or 8b. A PCR fragment of the expected size (806 bp) for a mature transcript containing exon 8a was detected in all stages of development as described in Section 3.3. Transcripts were easily detected in prepupal and pupal cDNA (Fig. 4B, top panel, lanes 4 and 5), perhaps reflecting a high abundance at these stages. PCR products containing exon 8b sequences were prominent in pupal cDNA (lane 5), but they were barely detectable in embryonic, late larval and prepupal cDNAs (lanes 1, 3, and 4), and were undetectable in early larval and adult cDNAs (lanes 2 and 6). These data indicate that the utilization of exons 8a and 8b is variable throughout the different stages of *Tribolium* development. The developmental stages at which transcripts with exon 8b accumulate are apparently much more restricted than those in which exon 8a transcripts are expressed.



	Embryos	Early larvae	Last larvae	Prepupae	Pupae	Adults
TcCHS1	+	tr	+	++	+++	+
E8a/E8b	tr/tr	tr/ND	+/tr	++/tr	++/++	+/ND
TcCHS2	tr	tr	++	ND	ND	+

ND = not detected. tr = trace detected.



	Pupae		Adults (0-3 d)		Adults (4-5 w)	
	M	F	M	F	M	F
TcCHS1	++	+	+	+	ND	ND
E8a/E8b	+/+	+/+	+/ND	+/ND	ND/ND	ND/ND
TcCHS2	ND	ND	tr	+	tr	+

ND = not detected. tr = trace detected.

Fig. 4. Developmental pattern of expression of *TcCHS1* and *TcCHS2* genes. RT-PCR was done with first strand cDNA synthesized from RNA isolated from insects at different developmental stages. Lane 1, embryos; lane 2, early larvae (penultimate instar); lane 3, last instar larvae; lane 4, prepupae; lane 5, pupae; and lane 6, adults. Two micrograms of total RNA were used for each reverse transcriptase reaction to generate the cDNA which served as the template for the PCR. This was followed by PCR amplification using two pairs of primers specific for *TcCHS1* (exon 6/exon 10) and *TcCHS2* (exon 6/exon 8) in panel A or a pair of primers specific for exon 8a or 8b of *TcCHS1* (exon 6/exon 8a or exon 6/exon 8b) designed to yield products of different sizes for each gene (or alternate exons) in panel B. Specificity and equivalent amplification of exon 8a- and exon 8b-specific products are demonstrated in bottom panel of B by setting up a reaction with equal amounts of *TcCHS1* (lane 7) and *TcCHS2* (lane 8) cDNAs as templates and the mixture (lane 9) of the same primers used in top panel of B. Panel C indicates the PCR products derived from pools of larvae (lane 1), young adults (lane 2), and mature adults (lane 3) that were either all males or all females using the same sets of primers used in panels A and B to distinguish between *TcCHS1* and *TcCHS2* transcripts and between the *TcCHS1* transcripts containing either exon 8a or 8b. The PCR products were subjected to electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. The tables below each figure summarize the results.

3.5. Comparison of the sequences of exons 8a and 8b of *TcCHS1*

Exons 8a and 8b of *TcCHS1* are both 177 bp-long and exhibit a nucleotide sequence identity of 63%.

The nucleotide sequence identities of exon 8a and 8b to the corresponding region in *TcCHS2* are 58% and 55%, respectively. Both exons code for 59-amino acids and include a transmembrane segment of 20 amino acids in the middle of the sequence. The protein sequences encoded by exons 8a and 8b have an amino acid sequence identity of 70% and a similarity of 85% (Fig. 5). The N-terminal sequences of these exon-encoded peptides are postulated to be extracellular and the C-terminal segment is predicted to be exposed to the cytoplasm. The transmembrane sequence is more highly conserved than the two flanking sequences. Even though TcCHS2 has a stretch of amino acid sequence in exon 6 equivalent to exon 8a- or 8b-encoded segments including a transmembrane segment, it has a much lower level of amino acid sequence identity with these segments (only 54% and 44%, respectively).

3.6. Association between the presence of the coiled-coil domain and alternate exons

An alignment of the amino acid sequences corresponding to exons 8a and 8b of *TcCHS1* and equivalent regions from several other insect CHSs is shown in Fig. 6. While there is substantial amino acid sequence identity among all of these sequences, the identity is greater within the two subgroups of sequences related to exon 8a or exon 8b of *TcCHS1* (Fig. 6, top and bottom panels, respectively). Table 2 indicates that there is a correlation between the presence of the coiled-coil region (as predicted by the Paircoil Program) that immediately follows the catalytic domain and the presence of alternate exons. Only insect genes that code for CHSs with the coiled-coil region immediately following the five-transmembrane span (5-TMS) region have alternate exons corresponding to the exons 8a and 8b of *TcCHS1* gene. The insect *CHS* genes, *TcCHS2*, *DmCHS2*, *AaCHS1* and *AgCHS1*, which do not encode a CHS with the coiled-coil region immediately following the 5-TMS region, do not have the alternate exons. Included in this list of genes containing alternate exons is the *LcCHS1* gene from *L. cuprina* whose genomic DNA sequence has not yet been published. However, the cDNA sequence of *LcCHS1* (Tellam et al., 2000) encodes a CHS protein that has a high probability of having a coiled-coil domain. Based on this and the alignment of the alternate exon-encoded protein region to other insect CHSs, we predict that this gene also will have alternate exons. A similar prediction that the *MsCHS1* gene has alternate exons was confirmed experimentally by PCR amplification and DNA sequencing of the region. The alternate exons from *M. sexta CHS1* also encode 59-amino acid-long stretches with sequence similarities to correspond-

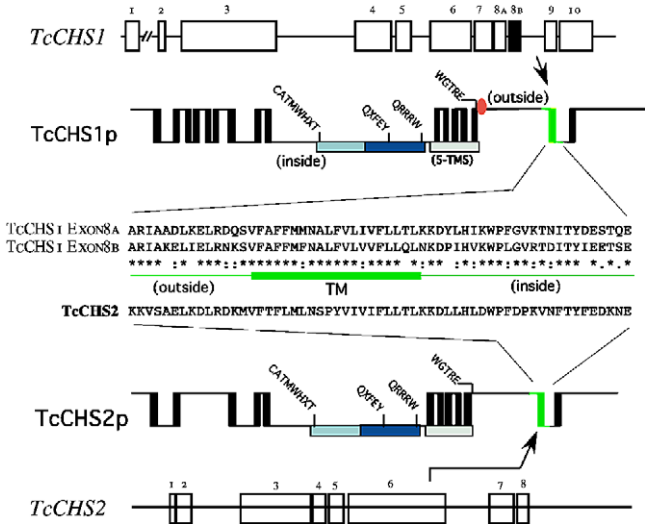


Fig. 5. Comparison of the transmembrane and flanking sequences of TcCHS1 encoded by exons 8a and 8b with the corresponding region in TcCHS2. Alignment of deduced amino acid sequences was made using ClustalW software (PAM250). In the alignments, identical amino acids are indicated by an asterisk, highly conserved amino acids by a colon, and less conserved amino acids by a period (see center of figure). The regions that were aligned are depicted in the exon–intron and transmembrane maps of each gene in the line drawings for the genes (*TcCHS1* and *TcCHS2*). Exons and introns are depicted as in Fig. 2. The ORFs begin in exon 2 of *TcCHS1* and in exon 1 of *TcCHS2*. Predicted transmembrane profiles were made using TMHMM (v. 2.0) (TcCHS1p and TcCHS2p). Transmembrane helices are indicated as vertical (thick) bars and regions predicted to be on the inside or outside of the plasma membrane by horizontal (thin) lines. For orientation, blocks of highly conserved amino acids are indicated. The probable catalytic domain is boxed; the darker shaded portion depicts the region with high sequence similarity to fungal chitin synthases and the lighter shaded portion with low sequence similarity to fungal chitin synthases. The transmembrane spans that may be important for extrusion of chitin through the plasma membrane are indicated as 5-TMS. The shaded oval in the TcCHS1 map identifies a predicted coiled-coil domain (Paircoil Program).

```
                    Extracellular          Transmembrane helix          Cytoplasm
TcCHS1a     ARIAADLKELRDQSVFAFFMMNALFVLIVFLLTLKKDYLHIKWPFGVKTNITYDESTQE
AgCHS2a     ARIAHDLKELRDSAVFGFIMINALFVLIVFLLQLNKDNIHVKWPLGVKTNITYDEATQE
DmCHS1a     ARIAKDLKELRDSSVFAFFMINALFVLIVFLLQLNKDNIHVKWPFGVRTNITYDESTQE
MsCHS1a     ARISRDLKELRDSSVFSFFMINALFVLIVFLLQLNKDNIHVKWPFGVKTNITYDEVSRE
LcCHS1      ARIAKDLKELRDSSVFFFFMVNALIVSIVFLLQLNKDNIHVKWPFGVRTNITYDESTQE
            ***: *******.:** *:*:***:* ***** *:** :*:***:**:******* ::*

                    Extracellular          Transmembrane helix          Cytoplasm
TcCHS1b     ARIAKELIELRNKSVFAFFMFNALFVLVVFLLQLNKDPIHVKWPLGVRTDITYIEETSE
AgCHS2b     ARIAVDLKELRNKSVFAFFMFNALFVLIVFLLQLNKDKLHIIWPLGVKTNITYDEVTAE
DmCHS1b     ARIASDLIELRNKSVFAFFMANALFVLIVFLLQLNKDKLHIIWPLGVKTNITYIEETSE
MsCHS1b     ARIAGDLLELRNKSVFAFVMFNALFILIVFLLQLNKDQLHVVWPLGVKTNITYIEETGE
            **** :* **********.* ****:*:********* :*: *****:*:*** * *.*
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Fig. 6. Alignment of the amino acid sequences of the regions encoded by exon 8a and 8b of *TcCHS1* with corresponding regions of other insect CHS sequences from data bases except *MsCHS1b* (unpublished data). Top panel: alignment of alternative exon-encoded regions of the insect and nematode CHS of class A (see Fig. 8). Bottom panel: alignment of alternative exon-encoded regions of the insect and nematode CHS of class B (see Fig. 8). The symbols are as in Fig. 5).

ing regions in TcCHS1 (Fig. 6; Hogenkamp et al., unpublished data).

3.7. *Mapping of TcCHS1 and TcCHS2 to Tribolium* chromosomes

TcCHS1 and *TcCHS2* were mapped to linkage groups 5 and 9, respectively, using SSCP analysis as described in Section 2.9 (at map positions 46.8/54 cM and 20.2/62.2 cM; Beeman et al., unpublished data). This result was unexpected because in the only two other insect species in which all of the *CHS* genes have been mapped, namely *D. melanogaster* and *A. gambiae*, the genes are linked. For *D. melanogaster*, they are positioned about 3 Mb apart on chromosome 3 and for *A. gambiae*, about 8 Mb apart on chromosome 2 (Gagou et al., 2002; results of TBLASTN search of *A. gambiae* genome database at NCBI).

3.8. *Southern blot analysis of T. castaneum genomic DNA for detection of CHS genes*

To support that there are only two *CHS* genes in the *Tribolium* genome, Southern blot analysis of genomic DNA was carried out using five different restriction enzymes and a radioactive probe amplified from the highly conserved catalytic domain *TcCHS1* fragment as outlined in Section 2. In each digest, only two bands could be seen, one corresponding to *TcCHS1* and the other corresponding to *TcCHS2*. The observed sizes, when compared with those that could be predicted from the *TcCHS* gene sequences, were in agreement (data not shown). No additional bands were detected under moderate stringency conditions. Hybridization of the probe to the other *CHS* gene (and to *Drosophila CHS* genes) was easily detected (Fig. 7) supporting our conclusion from the screening of the BAC library that there are only two genes for CHS in the *Tribolium* genome.

4. Discussion

We have identified several clones containing *CHS* genes of *Tribolium* from a screening of a BAC library.

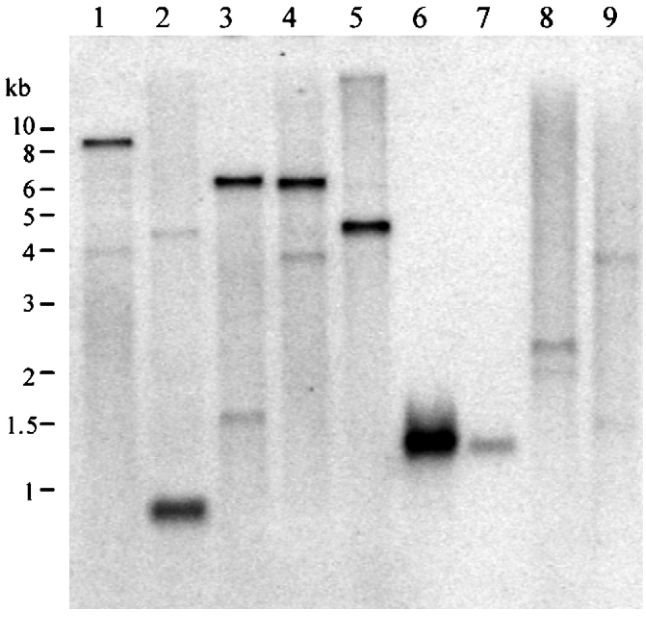

Fig. 7. Southern blot analysis of *Tribolium castaneum* and *Drosophila melanogaster* genomic DNA. *Tribolium* DNA (10 μg per lane) was digested with the five indicated restriction enzymes. Lane 1, *Ase*I; lane 2, *Cla*I; lane 3, *Hin*cII; lane 4, *Hin*dIII; lane 5, *Xba*I; lane 6, 100 pg of DNA fragment (1265 bp) corresponding to nucleotide positions 1474–2739 of *TcCHS1* cDNA sequence; lane 7, 100 pg of DNA fragment (1223 bp) corresponding to nucleotide positions 1435–2658 of *TcCHS2* cDNA sequence; lane 8, *Bam*HI digested *Drosophila melanogaster* DNA and lane 9, *Hin*dIII digested *Drosophila melanogaster* DNA. Hybridization was with the ^{32}P-labeled *TcCHS1* catalytic domain probe as described in Section 2.

Because the probe used for the screening was from the highly conserved region that encodes the catalytic domain of CHSs, we anticipated that it would hybridize under reduced stringency to different genes encoding CHS. Based on the strength of hybridization signals, length of PCR amplification products, and finally sequencing of the amplified DNAs from several BAC clones, we obtained evidence for the presence of only two *CHS* genes in *Tribolium*. The data from Southern blot analysis also indicate the presence of only two genes in *Tribolium*, which is consistent with the results obtained from studies of other insect species whose *CHS* genes have been identified. Search of the databases for the fully sequenced genomes of *D. melanogaster*, *A. gambiae*, and of the nematode *C. elegans* revealed the presence of two genes in each of these invertebrates. Even though only one *CHS* gene has been isolated from *L. cuprina* and *M. sexta*, it is likely that these insects also have two *CHS* genes (Tellam et al., 2000; Zhu et al., 2002; unpublished data from H. Merzendorfer, Osnabrueck University, and our laboratories).

The number and positions of introns in the two *TcCHS* genes are not conserved. Only two introns are in equivalent positions in the two *TcCHS* genes. Other exons are fused or split to form exons that differ in size between the two genes. The start of translation is in exon 2 of *TcCHS1* and in exon 1 of *TcCHS2*. At the protein level, the C-terminal domain of the TcCHS2 protein is considerably shorter than that of TcCHS1. In *Drosophila* and *Anopheles*, the *CHS1* and *CHS2* genes are closely linked (approximately 3 and 8 Mb apart in their respective species), indicating that the two arose via a tandem duplication and divergence from a single progenitor gene. Our discovery that the two genes are not linked in *Tribolium* suggests more extensive genome rearrangement in beetles than in flies, at least for this region of the genome. In the nematode, *C. elegans*, the *CHS* genes occur on separate chromosomes (*C. elegans* genome database, NCBI), like in *Tribolium*.

The two *CHS* genes characterized in this study encode proteins that have amino acid sequences closely related to those from other insects and nematodes, and more distant from those of fungi (Fig. 8). All members of the CHS-A class for which genomic DNA sequences are available have two alternate forms of the exon corresponding to exon 8 of *T. castaneum CHS1*. The proteins encoded by the CHS-A class are slightly larger than the CHSs of the CHS-B class and are predicted to have a coiled-coil region immediately following the 5-TMS region. No coiled-coil region was predicted for the CHS-B group of proteins. The coiled-coil regions are potential sites for protein–protein interactions and/or signals for vesicular trafficking (Melia et al., 2002). It is interesting that both alternate exons encode a

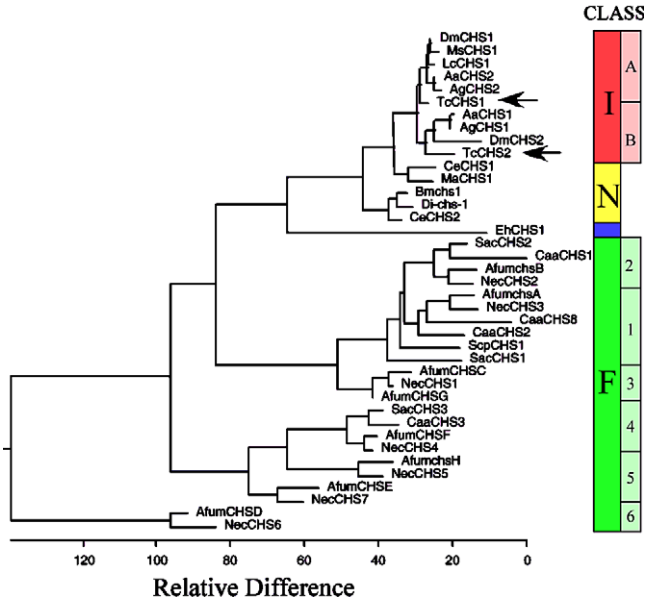

Fig. 8. Classification of insect, nematode and fungal CHSs based on relative amino acid sequence similarities. Sequences from Qx(F,Y)EY to QRRRW in the catalytic domains were aligned by ClustalW (PAM250) to generate the tree. CHSs of insects (I) were from *Aedes* (Aa), *Anopheles* (An), *Drosophila* (Dm), *Lucilia* (Lc), *Manduca* (Ms), and *Tribolium* (Tc); nematodes (N) were from *Brugia* (Bm), *Caenorhabditis* (Ce), *Dirofilaria* (Di), and *Meloidogyne* (Ma); fungi (F, shown in green; numbers refer to the six classes) were from *Aspergillus* (Afum), *Candida* (Caa), *Neurospora* (Nec), and *Saccharomyces* (Sac); and unclassified was the parasite, *Entamoeba* (Eh) shown in blue. Class assignment of fungal CHSs is based on Specht et al. (1996). Arrows indicate the two *Tribolium* CHSs.

highly conserved transmembrane domain, but have more variable sequences in the flanking regions that are predicted to be exposed to the external surface of the plasma membrane and to the cytosol. It is conceivable that these exposed domains interact with different regulatory molecules including those involved in vesicular trafficking. It is also worth noting that the corresponding region in TcCHS2 has fewer amino acid sequence identities with TcCHS1, even though it has a high similarity to the exon 8a- or 8b-encoded sequences (Fig. 5). Alternate splicing has been shown to alter the localization of a human plasma membrane Ca^{2+} ATPase from a basolateral location to a predominantly apical location (Chicka and Strehler, 2003). In that case, a cytosolically exposed 45 amino acid-long region was shown to be responsible for this change in distribution of the Ca^{2+} ATPase.

The qualitative differences observed in the developmental patterns of expression of the two *TcCHS* genes are consistent with the enzymes encoded by these two genes having different physiological functions. In embryos, only *TcCHS1* transcripts are detectable suggesting that this enzyme is involved in the synthesis of chitin associated with embryonic cuticle and mouth-

parts (Wilson and Cryan, 1997). The transcripts for *TcCHS2* are not detectable in embryos or pupae but are expressed in the last larval stage and in adults when there is active PM synthesis, suggesting that TcCHS2 is associated with formation of PM-associated chitin. Because we have isolated RNA from whole larvae (and not from gut and epidermal tissues free of cross-contamination from other tissues), we are unable to ascertain whether TcCHS2 is exclusively associated with midgut chitin synthesis. Tellam et al. (2000) investigated tissue specificity of expression of *LcCHS-1* (equivalent to *TcCHS1*) and concluded that this gene was expressed in the carcass (free of internal tissues) but not in cells of the midgut. Their in situ hybridization experiments also confirmed this tissue specificity of expression of *LcCS-1*. The data presented in this paper provide experimental support for the hypothesis that midgut chitin synthesis may be a major function of TcCHS2. Gagou et al. (2002) studied stage-specific expression of *CHS* genes in *Drosophila* using third instar larvae and prepupae, but they did not address the tissue specificity of expression of individual *CHS* genes. An increase in the level of *TcCHS1* mRNA was observed during pupation followed by a decline to undetectable levels during the adult stage. Thus, TcCHS1 very likely plays an important role in the formation of the pupal cuticle. On the other hand, *TcCHS2* transcript levels decline between the larval and pupal stages, and then reappear in adult insects. The developmental patterns of expression of *TcCHS* genes suggest that *TcCHS2* is not essential during the embryonic stage and possibly during the pupal stage. The high levels of *TcCHS2* mRNA in late larval stadia and in adults may be indicative of a role for the TcCHS2 protein in the production of the chitin-rich PM.

Developmental regulation of alternate exon usage appears to determine which form of the *TcCHS1* transcripts accumulate during different growth stages. In the embryonic stage, all of the CHS transcripts detected are derived from *TcCHS1* with either exon 8a or exon 8b. *TcCHS1* transcripts are undetectable in early instar larvae but are present in the last instar larvae and prepupae, and these contain predominantly exon 8a. The greatest amounts of *TcCHS1* transcripts (with either exon 8a or exon 8b) occur at the pupal stage when no *TcCHS2* transcript was detectable, suggesting the requirement of either a different type or larger amounts of CHSs at this stage. The appearance of exon 8b-containing transcripts is confined predominantly to the pupal stage, which is just about the time ecdysteroid titers are expected to decline based on analogy with hormonal levels in the tobacco hornworm (Riddiford, 1994). The finding that transcripts with exon 8a are expressed over a wider range of developmental stages than transcripts with exon 8b suggests that alternate splicing may be under hormonal and/or developmental control. However, this possibility needs to be investigated further using tissues lacking endogenous sources of hormones and neurotransmitters (Kramer et al., 1993). *DmCHS1* transcripts containing both exon 7a and 7b have been reported (NM_169052; *Drosophila* EST database: http://www.fruitfly.org/cgi-bin/annot/gene?kkv). We have found some evidence for the presence of *TcCHS1* transcripts containing both exons 8a and 8b, but they are very rare relative to transcripts containing only exon 8a or exon 8b (Arakane et al., unpublished data).

We have analyzed the DNA sequence of the region immediately upstream of the putative transcription start site of *TcCHS1* and *TcCHS2* for the presence of sequences with consensus ecdysone-response elements, EcRE (Palli and Retnakaran, 1999), and consensus sequences for the binding of transcription factors, BR-C and E74a (Thummel, 1996). In the approximately 1 kb promoter regions of both *TcCHS1* and *TcCHS2*, we have identified several putative EcRE and BR-C binding sequences that reasonably match the consensus sequences for these elements even though these matches were not perfect (data not shown).

Alternative splicing plays a major role in modulating gene function by expanding the diversity of expressed mRNA transcripts (Brett et al., 2000). While seeking to understand the repertoire of *CHS* genes and the proteins encoded by them in insects, we have obtained evidence for alternative splicing of pre-mRNA for CHS in *Tribolium*. *Drosophila*, *Anopheles* and *Manduca* also have *CHS* genes (CHS-A class, Fig. 8) that have alternate exons corresponding to *Tribolium* exon 8 and, therefore, the potential to form two CHS1 enzymes as a result of alternative splicing. The enzymes of the CHS-B class, on the other hand, are predicted to have only a single form. Alternate splicing to generate multiple isoforms of a serine protease inhibitor with different inhibitory specificities from a single gene with several alternate exons has been described in *M. sexta* (Jiang et al., 1996). Similarly, alternate splicing leads to different isoforms of tropomycin in *C. elegans* and the Pdp1 protein in *D. melanogaster* (Anyanful et al., 2001; Reddy et al., 2000). In this paper, we have provided experimental evidence that alternate exon usage does occur in *T. castaneum* for the purpose of chitin synthesis and that this process is developmentally regulated. In light of these findings, the contribution of alternative splicing to CHS diversity in other insect species and factors that control this process will need to be studied in the future. In addition, the significance of differences in biochemical properties and physiological functions of such alternate forms of CHSs in insects will be worthy of investigation.

6. Acknowledgments

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